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Intracellular transport, cell-surface exposure and release of recombinant Tamm-Horsfall glycoprotein

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Intracellular transport, cell-surface exposure and release of recombinant Tamm-Horsfall glycoprotein. Human Tamm-Horsfall glycoprotein (T-H), first described as the major urinary glycoprotein, is a glycosylphosphatidyl-inositol (GPI)-anchored membrane protein which mainly resides at the luminal face of cells of the thick ascending limb of Henle's loop (TAL) and early distal convoluted tubules of nephron. Since no human renal cell-line producing T-H is available, T-H cDNA was transfected in HeLa cells and a cell line was selected in which 95% of the cells stably expressed T-H, in order to elucidate the biosynthesis, mechanisms regulating the transport of T-H along the exocytic pathway, exposure at the cell surface and release in soluble form. Treatment of cells with an exogenous reducing agent results in a drastic delay in the conversion from precursor to mature T-H. Since the accumulating T-H-precursor carries glycans not yet processed by Golgi-mannosidases, we propose that the formation of a correct set of intrachain disulphide bonds is required for T-H exit out the endoplasmic reticulum. Even the treatment of cells with an inhibitor of GPI-anchor biosynthesis results in an intracellular accumulation of T-H precursor, loss of T-H localization into Golgi apparatus and reduced surface exposure. These results indicate that the GPI-anchor addition is necessary for T-H delivery to the cell-surface. The release rate of new synthesized T-H shows an initial lag time very likely depending on the time required for T-H surface exposure. A portion of released T-H appears to contain ethanolamine, a component of GPI anchor, indicating that, at least in HeLa cells, a GPI-specific phospholipase contributes to the T-H release. Exposure of cells to monensin and brefeldin A results in a loss of accumulation of T-H in the Golgi perinuclear region and a reduced delivery to the cell surface. Under monensin treatment an intermediate T-H form non-exposed at the cell surface is released in the medium, indicating that a soluble T-H may be produced inside the cell under conditions that alter the Golgi apparatus. If such an event occurs in polarized kidney cells, a T-H release from the basolateral face may be postulated, inasmuch as the GPI-anchor is an apical sorting signal. Since T-H is a powerful autoantigen, the accumulation of soluble T-H in the interstitium of TAL may cause the formation of immunocomplexes.

Human Tamm-Horsfall glycoprotein (T-H) has been first described as an urinary glycoprotein [1]. Indeed, T-H is the most abundant protein in normal human urine, being excreted in quantities of 50 to 100 mg per 24 hours [2]. In urine it occurs in form of large aggregates (M_r 6 × 10^6) constituted by a single glycoprotein of 80 kDa in unreduced sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which contains about 30% carbohydrate [reviewed in 3]. The peptide moiety of T-H, deduced by cDNA sequence, comprises 616 amino acids and 8 potential *N*-glycosylation sites [4, 5]. When RNAs isolated from about 150 different cell tissues were hybridized with a large probe for T-H RNA, only RNA from human adult kidney gives a positive signal, indicating that T-H is exclusively produced by kidney cells [4]. Immunoelectron microscopy has shown that T-H resides in the thick ascending limb of Henle's loop (TAL) and in the early distal convoluted tubules of nephron [6–8]. Rindler et al [9] demonstrated that T-H is a glycosylphosphatidylinositol (GPI)-anchored membrane protein and the addition of the GPI-anchor is very likely responsible for the delivery of the protein to the apical face of renal cells. On this basis, it may be assumed that urinary T-H is the released counterpart of membrane-bound protein. Indeed, many GPI-anchored proteins have been found in body fluids as soluble forms, very likely produced by the action of GPI-specific phospholipases or proteases [10]. Ours and other studies [11–14] on the carbohydrate structure of urinary T-H demonstrated that it contains exclusively *N*-linked glycans, mainly of polyantennary type largely sialylated and fucosylated, but high-mannose glycans ($Man_{5-7}GlcNAc_2$) have been consistently found in T-H isolated from pooled urine of various individuals [15–17].

We have previously studied [18] the biosynthesis and oligosaccharide processing of T-H permanently expressed by HeLa cells and have demonstrated that an 84 kDa precursor form is slowly converted into the mature glycoprotein with an apparent molecular weight of 97 kDa (SDS-PAGE in reducing conditions) very similar to that of T-H purified from human urine. The mass increase is due to the processing of the majority of high-mannose glycans to complex type glycans. The low maturation rate was suggested to be dependent on a lengthy retention of the precursor in the endoplasmic reticulum (ER). Owing to the high cysteine content of T-H, we postulated [18] that the rate limiting step for its export out ER compartment is the formation of a correct set of disulphide bonds that are in large part catalyzed by ER protein disulphide isomerase.

The physiological function of T-H glycoprotein has not been clarified to date. Speculations has been advanced mainly related to properties and structural features of urinary T-H. For instance, since the urinary T-H has a high gel-forming tendency [19, 20], it has been postulated that it takes part in the water impermeability of TAL [7, 21]. It is also been proposed that T-H plays a protective role towards pyelonephritogenic pathogens, such as *Escherichia*

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coli strains, which mediate the adhesion to the host cells by adhesins recognizing carbohydrate sequences of T-H, such as high-mannose glycans [18, 22]. T-H may inhibit the colonization of these pathogens in the renal mucosa in that the soluble form competes with that exposed at the plasma membrane.

Taking into accounts all these observations, it is important to ascertain mechanisms regulating the transport of T-H along the exocytic pathway, exposure at the cell surface and release as soluble protein. We investigated three steps of such a process: (*i*) the rate of exit from the endoplasmic reticulum (ER), very likely related to the formation of a correct set of disulphide bonds and incorporation of GPI-anchor; (*ii*) the role of *N*-glycan processing, occurring in the Golgi apparatus, in the delivery of T-H to the cell surface; (*iii*) the efficiency of release in soluble form. Since no human renal cell-line producing T-H is available, the investigation has been performed using transfected HeLa cells, which permanently express T-H [18].

METHODS

Materials

Urinary T-H was prepared as previously described [20]. Antiserum to T-H was raised in rabbits as described by Bloomfield et al [23]. High-mannose glycopeptides and endo-β-*N*-acetylglucosaminidase H (Endo-H) released oligosaccharides were prepared from urinary T-H as previously described [16]. [^{35}S]methionine (specific activity > 1000 mCi/mmol) was from ICN Flow (USA), [^{3}H]mannose (specific activity 25 mCi/mmol) was from Du Pont (New England Nuclear, Boston, MA, USA), and [^{3}H]ethanolamine (specific activity 17.9 Ci/mmol) from Amersham (Buckingham, UK). All culture reagents were supplied by Gibco (Grand Island, NY, USA). Solid chemicals and liquid reagents were obtained from E. Merck (Darmstadt, Germany) and Farmitalia Carlo Erba (Milan, Italy). Concanavalin A (ConA)-Sepharose was from Pharmacia (Uppsala, Sweden). Bio-Gel P-4 was from Bio-Rad (USA). Endo-H was from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Protein A-Sepharose, mannosamine (2-amino-2-deoxy D-mannose), monensin, brefeldin A (BFA), weat germ agglutinin (WGA) and β-octylglucoside were from Sigma Chemical Company (Munich, Germany).

Cell culture

HeLa cells were stably transfected with cDNA encoding for T-H as previously described [18]. From the transfected HeLa cells a cell line (HeLaA3+) was selected by limit dilution; in HeLaA3+ 95% of the cells stably expressed T-H, as quantitated by the cytofluorimetric analysis, using anti-T-H antiserum followed by fluorescein-conjugated anti-rabbit IgG. Cells were cultured in DMEM with 5% fetal calf serum and 0.2 mg/ml G418.

Radioactive labeling

The labeling experiments were performed in cells at the confluence. When [^{35}S]methionine was used as radioactive precursor, one hour before labeling the normal medium was replaced with DMEM containing 5% fetal calf serum but lacking methionine (labeling medium), and cells were then labeled for 15 to 60 minutes with labeling medium containing 100 μCi/ml [^{35}S]methionine. Cells were chased for variable times with a medium containing a tenfold excess of cold methionine and 10 μg/ml of cycloheximide (chase medium). When [^{3}H]mannose was used as a radioactive precursor the concentration of glucose in the labeling medium was lowered to 0.1 g/liter. The [^{3}H]mannose was at 100 μCi/ml and had been evaporated in order to not change the medium composition and salt concentration. After a 20-hour labeling period the cells were chased three hours in a medium with the usual concentration of glucose and in the presence of cycloeximide (10 μg/ml). [^{3}H]ethanolamine (50 μCi/ml) in serum free medium was added to the HeLaA3+ cell-monolayer and the metabolic labeling period was for 30 hours.

Isolation of radiolabeled Tamm-Horsfall glycoprotein

At the end of labeling period the medium was collected, the cell layer was washed with 20 mM sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl (PBS), and then lysated by PBS containing 1% NP-40, 1% deoxycholate, 0.2 mM TPCK and 0.2 mM TLCK (buffer A). The radioactive T-H from both cell extracts and media was immunoprecipitated by anti-TH rabbit antiserum adding 5 μl and 10 μl of antiserum to 100 μl of cell extract and to 1 ml of centrifuged medium, respectively. The (T-H) antibodies complex was incubated overnight at 4°C, then protein A Sepharose was added and incubated for 90 minutes. The protein A Sepharose was washed several times with buffer A, re-suspended in the buffer used for the electrophoresis containing 1% SDS. Samples were boiled for three minutes and subjected to SDS-PAGE (polyacrylamide, 8.5%). To immunoprecipitate T-H on the plasma membrane, the antibody was loaded on the HeLaA3+ monolayer and left at 4°C for 45 minutes. The antibody was then removed, and the monolayer washed several times with PBS. Finally the monolayer was lysed and coupled to protein A-Sepharose as described above.

Pronase digestion of [^{3}H]mannose labeled Tamm-Horsfall and fractionation of glycopeptides and oligosaccharides

The polyacrylamide gels were fluorographed to locate precursor and mature T-H. The areas of interest in the gel were excised, cut in small portions and subjected to Pronase digestion as previously described [18]. At the end of the digestion 1 ml of water was added, the mixture boiled for 10 minutes and centrifuged. The pellet was suspended in 1 ml of water and re-treated as above. The two supernatants were put together and lyophilized. In this way about 90% of the radioactivity present in the gel was recovered. Glycopeptides were fractionated on a column (1 × 75 cm) of Bio-Gel P-4 (400 mesh) equilibrated in 0.1 M pyridine/acetate buffer, pH 5 and on a column of ConA-Sepharose (0.5 × 10 cm) as previously described [24]. Recovery of radioactivity after chromatography on either column was 85 to 90%. The glycopeptides fractionated on Bio-Gel P-4 column as high-mannose species were subjected to Endo-H and isolated from the reaction mixture by Bio-Gel P-4 filtration. The radioactive oligomannosides obtained by Endo-H treatment were analyzed by HPLC as previously described [18].

Analysis of Tamm-Horsfall release

HeLaA3+ cells monolayer (3 cm ∅) were labeled for 30 hours with [^{35}S]methionine (50 μCi/ml) or [^{3}H]ethanolamine (50 μCi/ml), and at the end of the labeling period the medium was collected (30 hr labeling-medium). A new medium (serum free) was added to the cell monolayer and after 24 hours (24 hr

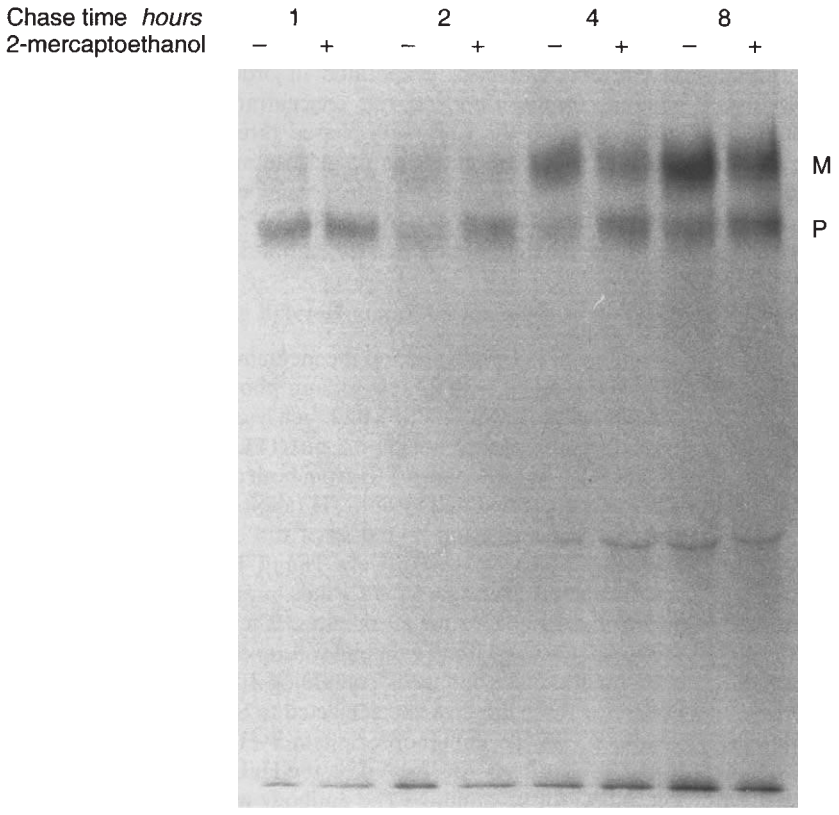

Fig. 1. Effect of 2-mercaptoethanol addition in the medium of transfected HeLa cells on Tamm-Horsfall glycoprotein (T-H) maturation. Cells were pulsed for 15 minutes with [^{35}S]methionine and chased for increasing periods. When present, 7 mM mercaptoethanol was added to the chase medium. T-H was immunoprecipitated from cell lysates and subjected to SDS-PAGE analysis as described in the text. M and P indicate the mature and precursor form of T-H, respectively.

medium) was collected and the cells harvested. T-H was immunoprecipitated from cell-lysate and the two media as described above and subjected to SDS-PAGE and fluorography. The bands of gel corresponding to mature T-H from cell lysate and media were excised. Since [^{3}H]ethanolamine-labeled T-H from the two media was not visualized by fluorography (Fig. 3B), the portions of gels parallel to that of mature T-H from cell-lysate were excised from the corresponding lanes. The gel slides were subjected to Pronase digestion as described above. The Pronase-digest was separated by centrifugation, and the residual gel slides were treated with H_2O_2 (5 hr at 50°C) and then eluted overnight with Soluene (Packard). Pronase-digest and Soluene-eluted samples were counted for radioactivity in β-scintillation counter.

Dot-blotting and immunodetection of Tamm-Horsfall glycoprotein

Conditioned medium (serum free) was collected at the indicated times and corresponding cell monolayers were washed three times with PBS. Cells were lysated with PBS buffer, pH 7.4, containing 60 mM octylglucoside and 5 mM EDTA. Serial dilutions of lysate and medium were performed with the same buffer containing 15 mM octylglucoside. One hundred microliter aliquots of dilutions were applied to the Hybond ECL membrane by a dot-blotting commercial apparatus, then the membrane was incubated with anti-T-H antiserum followed by anti-rabbit IgG conjugated to horseradish peroxidase and developed with the ECL detection system (Amersham) as recommended by the supplier.

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in PBS for one hour at 0°C (unpermeabilized cells) or with methanol pre-cooled at −20°C for 10 minutes (permeabilized cells). Unpermeabilized and permeabilized cells were incubated with pre-immune rabbit serum for 30 minutes at room temperature and then stained with anti-T-H antiserum (1:1000 dilution) followed by fluorescein-conjugated anti-rabbit IgG (1:300 dilution). Cells permeabilized and treated with pre-immune rabbit serum as described above were incubated for 30 minutes with WGA (10 μg/ml) and then with fluorescein-conjugated anti-WGA rabbit-antibodies (Sigma).

RESULTS

Maturation rate of Tamm-Horsfall glycoprotein in the presence of 2-mercaptoethanol

There is evidence that the addition of exogenous reducing agents, such as 2-mercaptoethanol and dithiothreitol (DTT), to the medium of living cells prevents the disulphide formation in newly synthesized proteins [25, 26]. To investigate the relevance of disulphide formation in the rate of T-H maturation, transfected HeLa cells were pulsed for 15 minutes with [^{35}S]methionine, and chased for increasing times in the presence or absence of 7 mM 2-mercaptoethanol. Figure 1 shows that at all chase times the ratio between precursor and mature T-H was in favor of the former when mercaptoethanol was present in the chase medium, indicating that the kinetics of maturation was decreased under the action

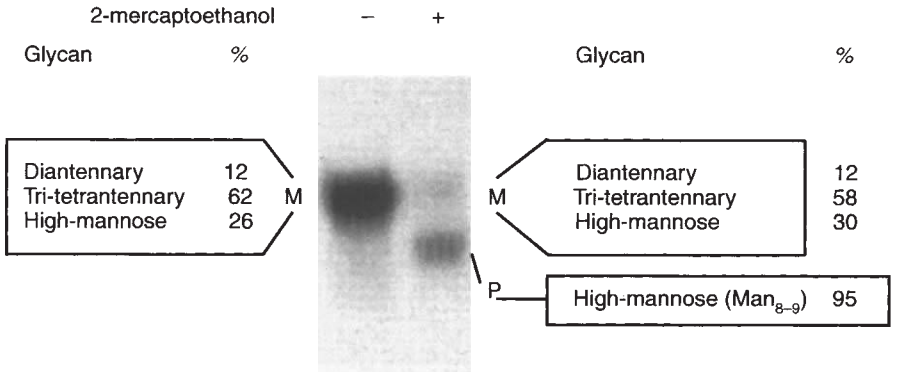

Fig. 2. Characterization of [^{3}H]mannose-labeled glycans of mature forms of T-H and of the precursor accumulating in 2-mercaptoethanol-treated cells. Cells were continuously labeled for 20 hours with [^{3}H]mannose in the absence or presence of 7 mM 2-mercaptoethanol. Fluorography of SDS-PAGE is shown in the insert. The portions of gel corresponding to the bands of mature T-H (M) and of precursor (P) were excised and subjected to Pronase-digestion. Glycopeptides and oligosaccharides were fractionated as described in the **Methods** section. The values are the percentage of the [^{3}H]mannose-derived radioactivity recovered in each type of glycopeptides or oligosaccharides. Data are the means of two different experiments.

of the reducing agent. No significant change was observed in the apparent molecular weight of mature protein synthesized by untreated and treated cells, suggesting that in the latter conditions the portion of glycoprotein that undergoes to maturation is efficiently processed by Golgi glycosyltransferases. To ascertain this point, untreated and 2-mercaptoethanol-treated cells were labeled with [^{3}H]mannose for 24 hours and the two mature T-Hs as well as the precursor accumulated in treated cells were isolated by immunoprecipitation and SDS-PAGE. Pronase glycopeptides from mature T-Hs were fractionated by ConA-Sepharose in order to ascertain the glycan-processing pattern [27] while pronase glycopeptides from precursor were digested by Endo-H and the reduced glycans analyzed by HPLC. A very similar ConA-Sepharose chromatographic profile of glycopeptides from the two T-H mature forms was observed. In both cases the majority of glycopeptides was eluted as polyantennary glycans (unretained by ConA-Sepharose column) and as diantennary glycans (eluted by 5 mM α-methylmannose; Fig. 2). On the contrary, all glycopeptides from T-H precursor accumulated in mercaptoethanol-treated cells strongly bound to ConA-Sepharose (eluted by 300 mM α-methylmannose) and after Endo-H treatment and HPLC analysis were eluted as $Man_{8-9}GlcNAc$, which are considered glycans not yet trimmed by Golgi mannosidases [28]. These results indicate that an exogenous reducing agent strongly delays the T-H exit out of the endoplasmic reticulum very likely because it interferes with the formation of disulphide bounds which are essential for a correct folding of the protein. 2-Mercaptoethanol does not affect significantly the subsequent glycans processing occurring into the Golgi apparatus.

Release of newly synthesized Tamm-Horsfall glycoprotein

HeLaA3+ cells were pulsed for one hour with [^{35}S]methionine, chased for increasing times and, at the end of each chase time lysates and media were immunoprecipitated and subjected to SDS-PAGE analysis. After a chase of eight hours, when the conversion from the precursor to the mature form was completed, the T-H was detectable in the medium and its release increased with the chase period (Fig. 3A). The initial lag time in the release very likely corresponded to the period required for the transit along the exocytic pathway and exposure of mature glycoprotein at the plasma membrane.

To investigate how membrane-bound T-H is released, particularly if proteases or a GPI-specific phospholipase were responsible for the cleavage, HeLaA3+ cells were metabolically labeled (30 hr) with [^{3}H]ethanolamine, a component of GPI-anchor (Fig. 4). Fluorography of SDS-PAGE visualized [^{3}H]ethanolamine-labeled T-H from cell lysate, but not from the conditioned medium collected at the end of the labeling period and 24 hours later (Fig. 3B). On the contrary, when cells were similarly labeled with [^{35}S]methionine, and the medium collected at the same times, the fluorograpy of SDS-PAGE clearly visualized the [^{35}S]methionine-labeled T-H released in the medium (Fig. 3B). Since [^{3}H]labeled-proteins are more hardly detectable by fluorography than [^{35}S]-labeled proteins, these results did not prove that the released T-H was devoid of ethanolamine. To clarify this point, the gel slides corresponding to various T-Hs separated by SDS-PAGE as shown in Figure 3B were analyzed by β-scintillation counting. As detailed in the **Methods** section, radioactive T-Hs were removed from the gel slides first by pronase treatment, which usually results in the solubilization of 90 to 95% of peptide components [18], and then by Soluene. Considering that there are 15 methionine residues versus 1 ethanolamine per T-H mole, the much higher radioactivity recovered in [^{35}S]methionine-labeled T-H samples was expected. As shown in Table 1, the percentage of labeled T-H released from cell-bound [^{3}H]ethanolamine-labeled T-H was significantly lower than that released from cell-bound [^{35}S]methionine-labeled T-H. The data of Table 1 have allowed us to calculate that 15 to 30% of the total T-H released contains [^{3}H]ethanolamine, indicating that a GPI-specific phospholipase contributes to the T-H cleavage.

Relative proportion of cell-bound and released T-H

The total amount of T-H released by HeLaA3+ cells in the medium relative to that cell-bound was determined by the ECL dot-blot immunodetection. A preparation of urinary T-H purified to homogeneity was used as standard [20]. Figure 5 shows a representative dot-blotting experiment of T-H recovered in cell lysates and media collected at increasing times of culture. As expected, a time-dependent release of T-H was found. The ratio between the T-H amount quantitated in 24-hour conditioned medium and that in corresponding cell lysate was 0.18 (mean of 2 different experiments), a value close to that found for T-H release from [^{35}S]methionine-labeled T-H (Table 1). When lysates and conditioned media of untransfected HeLa were analyzed with this procedure no positive signal could be detected (results not shown).

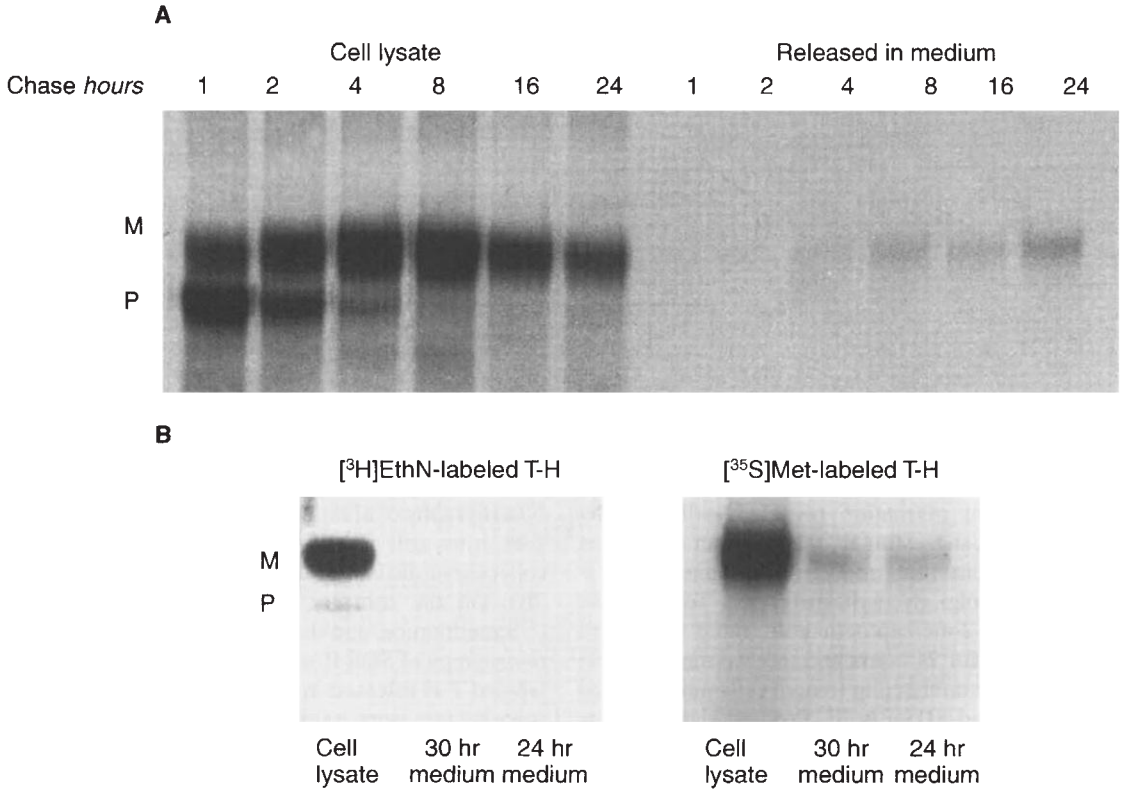

Fig. 3. Release of newly synthesized T-H. (*A*) HeLaA3+ cells were pulsed for one hour with [^{35}S]methionine and chased for increasing periods as indicated. T-H was immunoprecipitated from cell lysates and media and then subjected to SDS-PAGE. (*B*) HeLaA3+ cells were labeled with [^{35}S]methionine ([^{35}S]Met) or [^{3}H]ethanolamine ([^{3}H]EthN) for 30 hours and T-H was immunoprecipitated from cell lysates and the two conditioned media collected either at the end of labeling period (30 hr medium) or at further 24 hours (24 hr medium) and then subjected to SDS-PAGE as described in the text. Fluorography of gel corresponding to [^{3}H]ethanolamine ([^{3}H]EthN)-labeled T-H was exposed for seven weeks.

Effect of mannosamine on the maturation and release of T-H glycoprotein

A bulk of evidence indicates that GPI-anchor attachment occurs at the luminal side of endoplasmic reticulum compartment [29]. This reaction is a transamidase type of process where the original hydrophobic stretch of C-terminal amino acids is cleaved and replaced by a preformed GPI-anchor via the terminal amino function of ethanolamine of GPI unit [10]. The more conserved glycan structure of GPI anchor comprises the Manα1,2Manα1, 6Manα1,4GlcN-R sequence (Fig. 4), and it has been observed that when cells were treated with mannosamine there is a dramatic impairment of the GPI-anchor biosynthesis and a reduced exposure of GPI-anchored glycoproteins at the cell-surface [30–32].

We examined the effect of mannosamine-treatment on the biosynthesis, surface exposure and release of T-H. When HeLaA3+ cells were pulsed for one hour with [^{35}S]methionine and chased for 20 hours in the presence of mannosamine, SDS-PAGE analysis of the immunoprecipitate from cell lysate revealed a large accumulation of the precursor, and a medial mobility of mature T-H slightly faster than that produced by untreated cells (Fig. 6). The accumulation of T-H precursor could be related to the inefficient routing of T-H to the Golgi apparatus because of the inhibited biosynthesis of GPI-anchor [33], while the lower apparent molecular weight of mature T-H is very likely dependent on

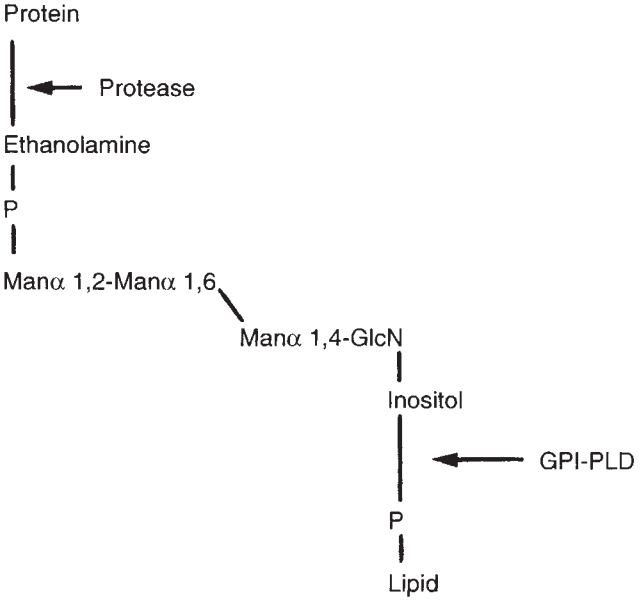

Fig. 4. Minimal structure of a GPI-anchor. Abbreviations are: P, phosphodiester group; Man, mannose; GlcN, glucosamine; GPI-PLD, GPI-specific phospholipase D. Arrows indicate the site of protease and GPI-PLD action.

Table 1. Release analysis of [^{3}H]ethanolamine (EtN)- or [^{35}S]methionine (Met)-metabolically-labeled T-H

Tamm-Horsfall glycoprotein (mature form)	Pronase digest	Soluene eluted	Total	% of Cell lysate
[^{3}H]EtN-T-H from cell-lysate	1093	3414	4507	—
[^{3}H]EtN-T-H from 30 hr labeling-medium	82	72	154	3.4
[^{3}H]EtN-T-H from 24 hr medium	52	44	96	2.1
[^{35}S]Met-T-H from lysate	30420	2003	32423	—
[^{35}S]Met-T-H from 30 hr labeling medium	3560	255	3815	11.7
[^{35}S]Met-T-H from 24 hr medium	4410	296	4706	14.5

The conditions of labeling and the β-scintillation counting of T-H samples, separated by SDS-PAGE as shown in Fig. 3B, are as described in the text. The values are means of two separate experiments.

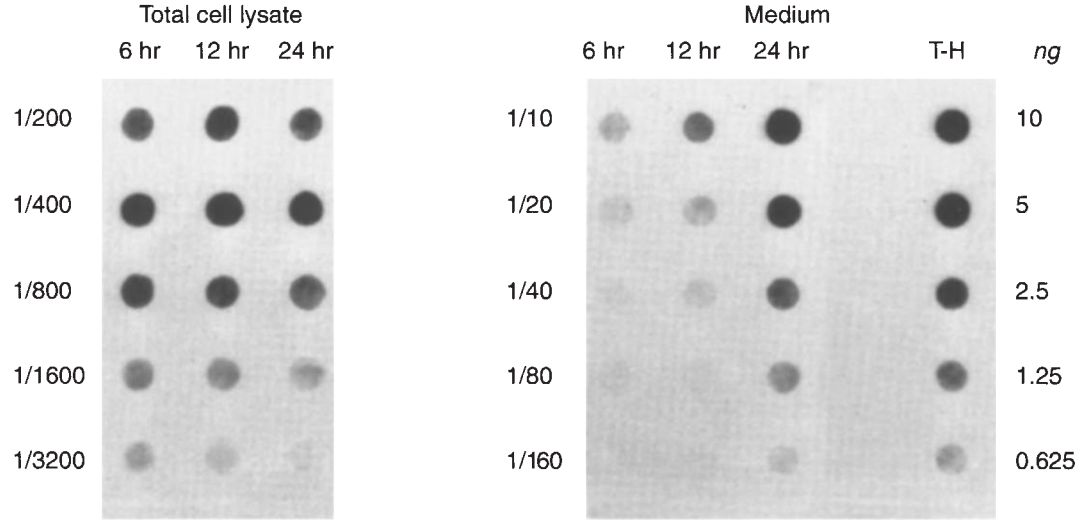

Fig. 5. Dot-blotting and ECL-immunodetection of T-H in cell lysate and medium of HeLaA3+ cells. Serial dilutions of lysate and medium were performed as described in the text and 100 μl of each dilution were applied to the Hybond ECL membrane. The membrane was incubated with anti-T-H antiserum followed by horseradish peroxidase anti-rabbit IgG and developed with the ECL detection system.

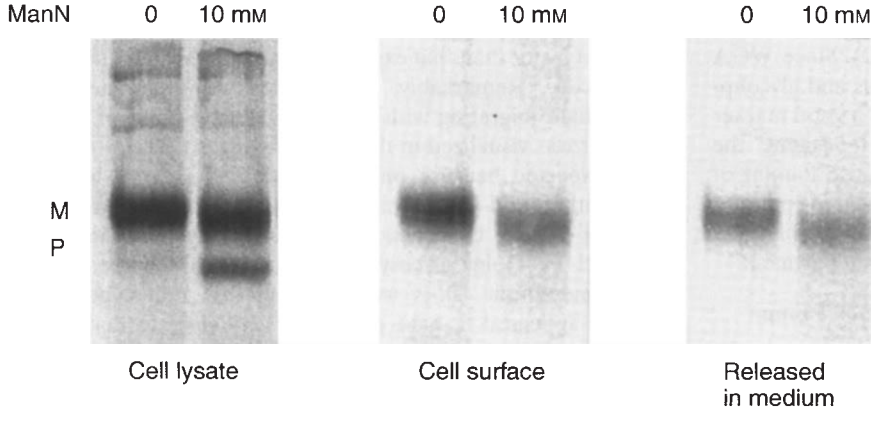

Fig. 6. Effect of mannosamine (manN) on the maturation, cell exposure and release of T-H. HeLaA3+ cells were labeled for one hour with [^{35}S]methionine and chased for 20 hours. When present, mannosamine (10 mM) was added to the medium three hours before the labeling period and it was present during the pulse and chase time. T-H was immunoprecipitated and isolated from the cell lysate, cell-surface and medium as described in the text. M and P indicate the mobility of mature and precursor T-H.

the altered *N*-glycosylation also produced by mannosamine treatment [34]. Both in untreated and mannosamine-treated conditions, only mature T-H appeared to be exposed at the cell surface and released in the medium (Fig. 6). The amount and apparent molecular weight of both cell-surface and released T-H were similarly reduced under mannosamine treatment, strongly suggesting that the cleavage producing the soluble form is an event occurring at the cell surface. Even in experiments in which the treatment with mannosamine was extended to 24 hours, we did not observed any degraded T-H-products either in cell lysates or in the medium. Immunofluorescence microscopy of unpermeabilized cells confirmed that mannosamine treatment drastically reduced T-H localization at the cell-surface (Fig. 7 A, B). In control permeabilized cells either WGA or anti-T-H antibodies

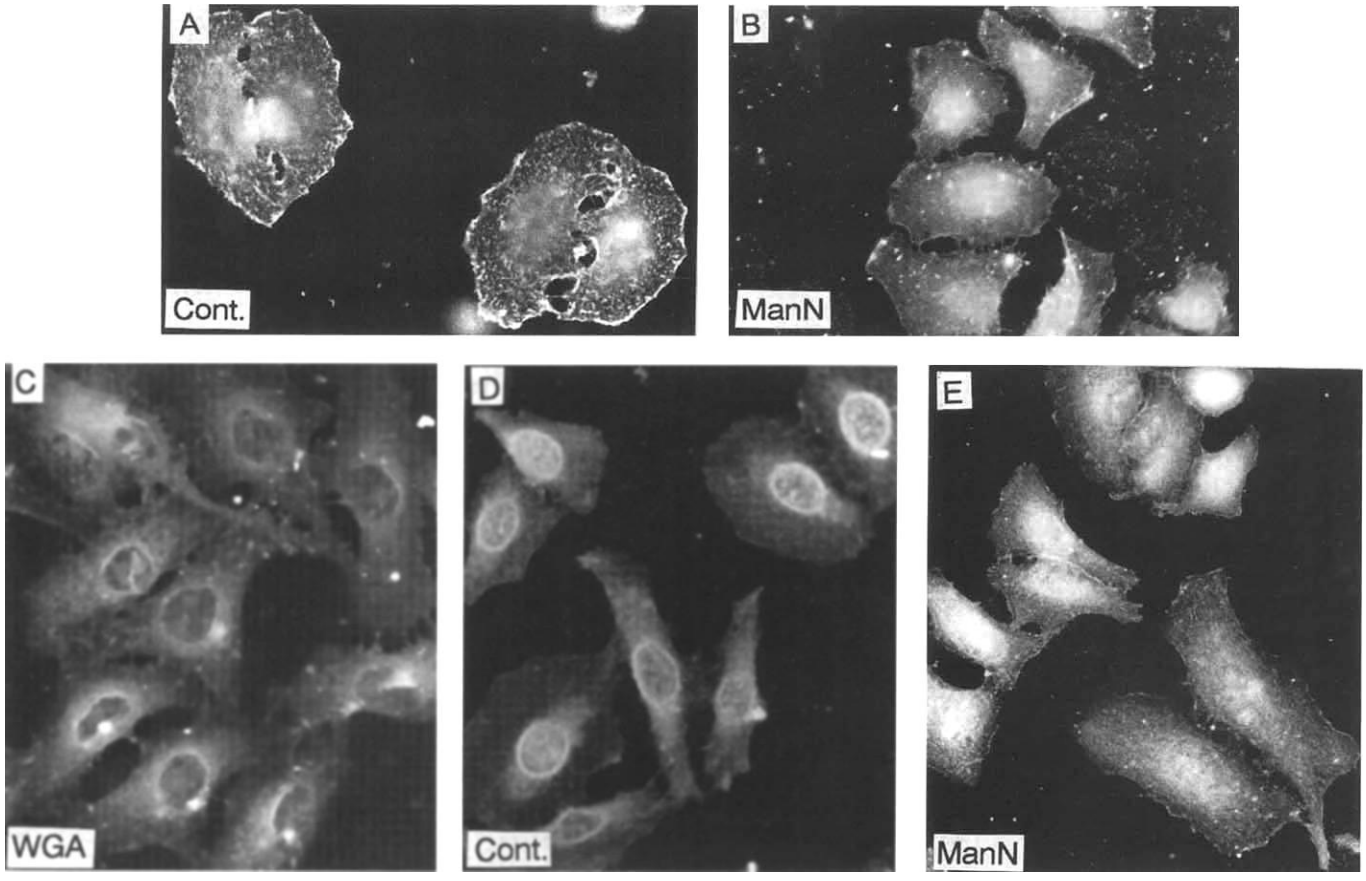

Fig. 7. Immunofluorescence localization of T-H in mannosamine-treated or untreated HeLaA3+ cells. The cells were grown in coverslips and treated or not with 10 mM mannosamine for 20 hours. Then the cells were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in PBS for one hour at 0°C (*A and B*: unpermeabilized cells) or with methanol for 10 minutes (*C, D, and E*: permeabilized cells). Unpermeabilized and permeabilized cells were incubated with pre-immune rabbit serum for 30 minutes at room temperature and then stained with anti-T-H antiserum (A, B, D and E) or WGA (C) followed by fluorescinated specific antibodies as described in the text.

illuminated the perinuclear structures (Fig. 7 C, D). Since WGA is a lectin that detects fully processed glycoproteins and glycolipids in that binds terminal sialic acid of glycans, it is a good marker of Golgi structures [35]. Under mannosamine treatment, the perinuclear localization of T-H was lost and an intense staining of the reticular network of endoplasmic reticulum became apparent (Fig. 7E), indicating that mannosamine treatment does inhibit the exit out ER of T-H and its routing to the Golgi compartment.

Effect of monensin and brefeldin A on the release of Tamm-Horsfall glycoprotein

Monensin is a monovalent carboxylic ionophore that largely affects the function of Golgi compartment and inhibits the processing of *N*-glycans from high mannose to the polyantennary type [36]. We investigated the effect of monensin on the maturation and release of T-H to ascertain the relevance of full processing of *N*-glycans in facilitating the T-H transport and exposure at the cell surface. As expected, under monensin treatment the maturation of T-H was radically altered: T-H from cell-lysate migrated as a broad band with a medial mobility much faster than that of T-H produced by untreated cells (Fig. 8). At the cell-surface only a narrow band was visualized with a mobility corresponding to the upper portion of the band visualized in the cell lysate, but faster than that exposed to the surface of mannosamine treated cells. Remarkably, under monensin treatment a T-H intermediate migrating with a mobility close to that of the T-H precursor was visualized in the medium (Fig. 8). The latter result was unexpected because only the mature T-H form had been consistently found in the cells medium, and this indicates that a soluble T-H may be produced before the glycoprotein is fully processed by Golgi glycosyltransferases and delivered to the plasma membrane. It is worth noting that the soluble form although appeared to have a mobility very close to that of T-H precursor did not appear to be degraded.

Treatment of cells with brefeldin A (BFA) has been demonstrated to induce a rapid redistribution of Golgi proteins into the endoplasmic reticulum (ER) compartment and a consequent impairment of the glycosylation mediated by distal compartments of Golgi apparatus [37]. As expected, BFA-treated cells accumulated a T-H form with a faster mobility of fully processed mature T-H, in the medium such a form was practically absent, while a faint smear of degraded products was visualized (Fig. 8), suggesting that intracellular T-H is degraded when Golgi apparatus fuses with the ER. In agreement with the inefficient maturation process of T-H indicated by SDS-PAGE analysis, the immunofluorescence microscopy of monensin- and BFA-treated cells showed a

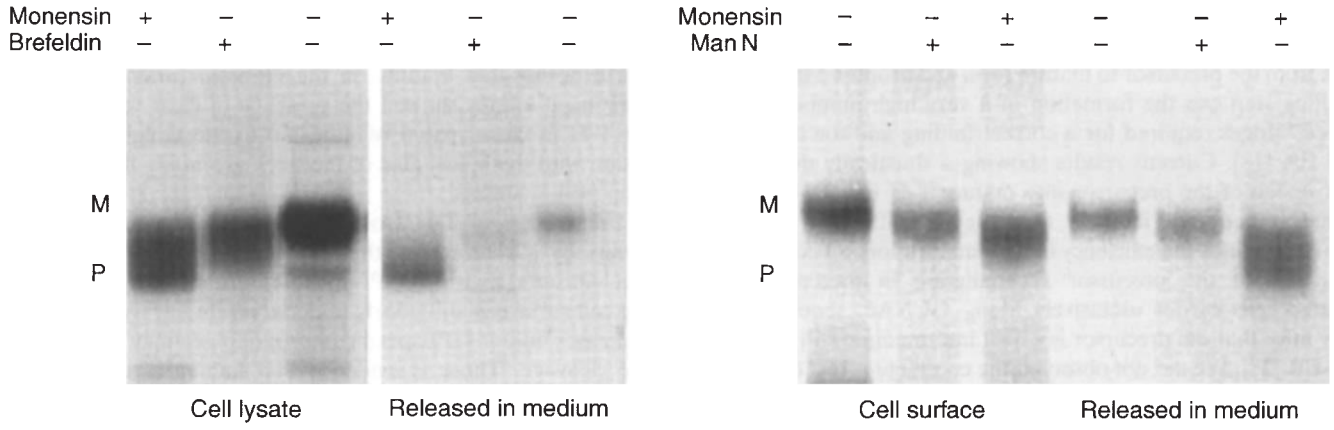

Fig. 8. Effect of monensin and brefeldin on the maturation, cell exposure and release of T-H. HeLaA3+ cells were pulsed for one hour with [^{35}S]methionine and chased for four hours. Monensin (10 μM) or BFA (10 μg/ml) were added to the medium at the chase time. The procedure of T-H isolation was as indicated in the legend of Figure 6.

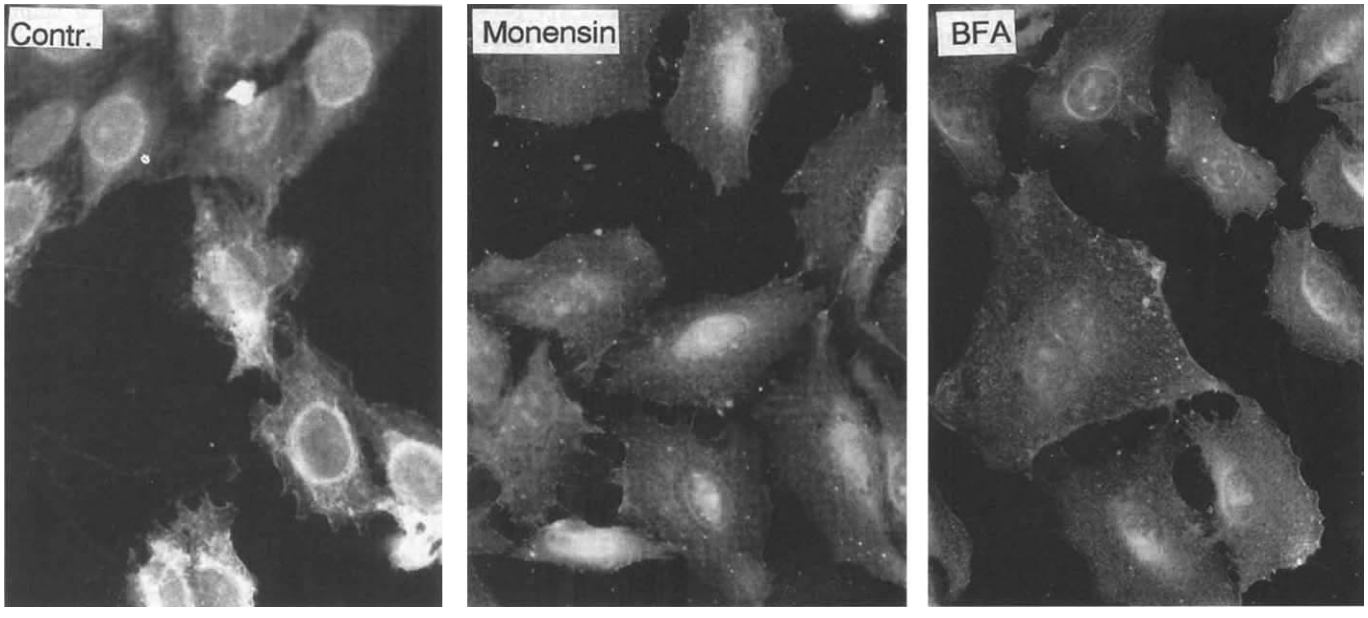

Fig. 9. Immunofluorescence localization of T-H upon monensin and BFA cell treatment. Monensin (10 μM) or BFA (10 μg/ml) were added to the cell medium and left for two hours. After washing, cells were fixed with methanol (permeabilized cells) and T-H was localized as described in the legend of Figure 7.

strongly reduced perinuclear staining and dispersal of T-H throughout the cell cytoplasm (Fig. 9).

DISCUSSION

There are several demonstrations that a correct folding is necessary for the export out the endoplasmic reticulum (ER) of both soluble and membrane-bound glycoproteins [reviewed in 38]. In the case of glycoproteins containing disulphide bonds the formation of a correct set of disulphides is thought to occur in ER because it provides a sufficiently oxidizing environment and there is a high content of protein disulphide isomerase. T-H contains 48 cysteine residues/mol prevalently distributed in the N-terminal portion of the peptide (37 residues in the first 295 aa). No free sulphydryl groups have been detected in T-H and all cysteine residues have been proposed to be involved in intrachain disulphide bonds [39]. Indeed, the mobility of T-H in SDS-PAGE in reducing conditions does not appear to be faster than that observed in unreducing conditions. In fact, in unreducing conditions the apparent molecular weight of T-H, either the urinary or mature membrane-bound form manufactured by HeLaA3+ cells, decreases from 96 to 80 kDa. Such a decrement is very likely dependent on the large degree of polypeptide constrains imposed by intrachain disulphide bonds [40]. For some proteins the folding and oligomerization occur cotranslationally and in few minutes the protein is exported out of the ER, but for other proteins the half time of permanence in the ER is of several hours [41]. The differences in the folding rate have been related to the nature of polypeptide backbone rather than to the expression level [42].

Previously we observed that the biosynthesis of T-H permanently expressed by HeLa cells was characterized by a low conversion rate from the precursor to mature form and proposed that the rate limiting step was the formation of a very high number of disulphide bridges required for a correct folding and for the exit out the ER [18]. Current results showing a drastically delay in the conversion of the precursor into mature T-H in cells exposed to 2-mercaptoethanol indicate that the formation of disulphide bonds regulates the efficiency of T-H maturation. Since the glycan structure of the precursor accumulating in mercaptoethanol treated cells carries exclusively $Man_{8-9}GlcNAc_2$ sequences, one may infer that the precursor is a T-H intermediate still residing in the ER [28]. We did not observed the complete arrest of T-H into the ER as it has been observed for some proteins synthesized by cells exposed to DTT [25], probably because 2-mercaptoethanol is a milder reducing agent than the former. Interestingly, the portion of T-H that exits out of the ER in mercaptoethanol-treated cells appears to be processed by Golgi glycosyltransferases, indicating that at least the glycosylation function of the Golgi apparatus is poorly affected by exogenous reducing agents.

Several conditions interfering with the GPI attachment process, such as those preventing the cleavage of the C-terminus signal or the biosynthesis of GPI-anchor, were found to inhibit the delivery of GPI-anchored proteins to the cell surface [30, 33, 43]. By using mannosamine as an inhibitor of GPI biosynthesis we found that the addition of GPI-anchor is also required for T-H delivery to the cell surface. In contrast to a previous result [44], no degradation of intracellularly accumulated T-H was observed in mannosamine-treated HeLaA3+ cells, suggesting that such an event is protein- and cell-dependent. In untreated cells, intracellular T-H appears to be mainly located in the Golgi apparatus. When [^{3}H]mannose-labeled glycopeptides isolated from intracellular mature T-H were fractionated on DEAE-Sephacel chromatography they were entirely separated as di- and trisialylated glycopeptides (results not shown). Altogether these observations support the notion that mature T-H accumulates in the distal compartments of Golgi apparatus before being exposed at the cell surface. Various signal functions have been assigned to the GPI-anchor, including the facts that GPI-anchored proteins associate with Golgi glycosphingolipids to form microdomains in the *trans*-Golgi network and that this association facilitates the sorting of GPI-proteins to the apical cell surface [45]. Recently [46], GPI-anchored proteins have been described to cluster in plasma membrane conformations known as caveolae, and it has been proposed that the clustering of GPI-proteins with specific ligands in caveolae may be a crucial event in promoting specific functions at the cell surface.

Interestingly, T-H from rat shows 53% identity and 86% similarity with rat GP-2 the major glycoprotein of zymogen granule membranes of the exocrine pancreas [47]. GP-2 is also a GPI-anchored proteins and, similarly to urinary T-H, the GP-2 soluble form from the pancreatic juice shows ion-dependence and pH-induced self-association [47]. Leblond et al [48] showed that zymogen aggregation is strongly affected by the presence of GP-2 even in an acidic environment similar to that believed to exist in *trans*-Golgi network and proposed that the co-aggregation of the GP-2 and zymogen facilitates the routing of latter towards the regulated secretory pathway. One may ask if T-H accumulated in the distal compartments of the Golgi apparatus has a function of carrier of molecules that must be sorted to the apical face of renal cells in which T-H accumulates. To answer to this question an investigation must be done in kidney cells producing T-H. It is worth noting that a study on the ultrastructural localization of T-H in rat kidney showed the presence of clear vesicles positive for T-H in close spatial relationship to the Golgi stacks and in fusion with the apical face of the thick ascending limb of Henle's loop [49].

The release of T-H from transfected HeLa cells very likely occurs by a cleavage of that exposed at the cell surface. The mechanism(s) by which GPI-anchored proteins are released from the cell surface is still poorly understood. In eucaryotic cells both proteases and/or GPI-specific phospholipases may be involved in the cleavage. There is evidence that human serum contains a phospholipase D able to cleave GPI-anchored proteins [50], and recently in HeLa cells a cell-associated GPI-specific phospholipase D has been reported by Metz et al [51]. These authors observed that the decay accelerating factor, which is also a GPI-anchored protein, is secreted by [^{3}H]ethanolamine- and [^{3}H]inositol-labeled HeLa cells and contains both radiolabeled components. They calculated that about 40% of the molecules are released via phospholipase D. When we performed a similar experiment with [^{3}H]ethanolamine as the labeled precursor, a lower percentage of [^{3}H]ethanolamine-labeled T-H was found in the medium relative to the total T-H released. The scarce accessibility of membrane-bound T-H to the cell-associated GPI-specific phospholipase D may explain our result. On the other hand, these observations cannot be extended to explain the manner in which T-H is released in urine by kidney cells of TAL, in that the occurrence and the level of a GPI-specific phospholipase D in renal cells producing T-H have not been investigated. The cloning of T-H cDNA in kidney cells, such as MDCK, could be a good model to face the problem.

Current results show also that, when functions of Golgi apparatus are altered by monensin, a cleavage of T-H may occur before the delivery of the glycoprotein to the cell surface. This result may be relevant in understanding the role of T-H in some pathological conditions of kidney. T-H is a powerful autoantigen [52], but in physiological conditions the exclusive release from the apical face of tubular renal cells very likely prevents the immunological response to T-H. In humans, T-H deposits in the interstitium of TAL have been demonstrated to accompany tubulointerstitial diseases [53] and antibodies to T-H have been observed in the serum of patients with pyelonephritis and reflux nephropathies [54, 55]. Recently [56, 57], a proinflammatory potential of T-H has been proposed in that it triggers some neutrophil functions. Various kinds of cell damage may result in the accumulation of T-H in the interstitium, such as necrosis of TAL cells or loss of their apical/basolateral polarization. Since the GPI-anchor is thought to be the sorting signal to the apical membrane, even the cleavage of the GPI-anchor before T-H delivery to the cell surface may be a way by which T-H is excreted by the basolateral face. Therefore, the accumulation of soluble T-H in the interstitium of TAL, which causes the formation of immunocomplexes and inflammatory process, may also occur via perturbation of Golgi apparatus functions.

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APPENDIX

Abbreviations are: T-H, Tamm-Horsfall glycoprotein; GPI, glycosylphosphatidyl-inositol; TAL, thick ascending limb of Henle's loop; ER, endoplasmic reticulum; BFA, brefeldin A; ManN, mannosamine.

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